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Research paper

Neutrophil function of neonatal foals is enhanced *in vitro* by CpG oligodeoxynucleotide stimulation

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ABSTRACT

Rhodococcus equi is an intracellular bacterium that causes pneumonia in foals and immunocompromised adult horses. Evidence exists that foals become infected with *R. equi* early in life, a period when innate immune responses are critically important for protection against infection. Neutrophils are innate immune cells that play a key role in defense against this bacterium. Enhancing neutrophil function during early life could thus help to protect foals against *R. equi* infection. The objective of our study was to determine whether *in vitro* incubation with the TLR9 agonist CpG 2142 would enhance degranulation and gene expression of cytokines and Toll-like receptor 9 (TLR9) by neutrophils collected from foals at 2, 14, and 56 days of life, and to determine whether these stimulated responses varied among ages. Neutrophil degranulation was enhanced at all ages by *in vitro* stimulation with either CpG alone, *R. equi* alone, or in combination with either *R. equi* or N-formyl-methionyl-leucyl-phenylalanine (fMLP) ($P < 0.05$), but not by *in vitro* stimulation with fMLP alone. There were no significant differences among ages in CpG-induced cytokine expression, except for IL-12p40, which was induced more at 56 days of age than on days 2 or 14. Collapsing data across ages, CpG 2142 significantly ($P < 0.05$) increased IL-6 and IL-17 mRNA expression. We concluded that *in vitro* stimulation of foal neutrophils with CpG enhances their function by promoting degranulation and inducing mRNA expression of IL-6 and IL-17, regardless of age.

1. Introduction

Neonatal foals are susceptible to microbial infections, notably to the intracellular bacterium *Rhodococcus equi*, which survives and replicates in macrophages and causes severe pneumonia in foals (Meijer and Prescott, 2004). Although effective treatments exist, vaccines are currently lacking, and *R. equi* infection remains as an important cause of disease and death in foals (Cohen and Giguère, 2009; Cohen and Martens, 2007; Stratton-Phelps et al., 2000). Many foals appear to be infected during the first 2 weeks of life (Chaffin et al., 2008; Horowitz et al., 2001). At this age, foals rely heavily on innate immune responses because their adaptive immune system is naïve (Flaminio et al., 2000). Neutrophils are the most abundant effector cells of innate responses in blood, and provide a crucial link between innate and adaptive immunity: cytokine and chemokine expression by these cells helps to recruit and activate other effector cells of the immune system (reviewed in Denkers et al., 2003). The importance of neutrophils in protecting against *R. equi* infections has been demonstrated in mice (Martens et al., 2005), and

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observational data from foals indicate neutrophils play a protective role (Chaffin et al., 2004).

The protection offered by neutrophils to foals, however, could be compromised during early life. Age-dependent impairment in neutrophil function has been associated with neonatal susceptibility to infection in other species (Koenig and Yoder, 2004). The situation for foals is somewhat unclear. Some studies indicate that the function of foal neutrophils is similar to that of adults. In contrast, neutrophils from foals were reported to have functional impairment relative to that in adults, reflected by the decreased bactericidal activity and serum opsonic capacity (Grondahl et al., 1999; Martens et al., 1988; McTaggart et al., 2001), and cytokine gene expression changes with age in neutrophils of neonatal foals (Nerren et al., 2009a). If protection offered by neutrophils to foals is compromised during early life, it is plausible that altering functional responses of neutrophils in newborn foals might help to protect them against infection caused by *R. equi*.

Previously, our laboratory has demonstrated that foal neutrophils express TLR9 mRNA, and that cytosine-phosphate guanine oligodeoxynucleotides (CpG-ODNs) can activate cytokine expression and reactive oxygen species (ROS) production by foal neutrophils, both in purified preparations of neutrophils and in neutrophils in contact with other leukocytes (Liu et al., 2009a). However, we did not examine responses to CpG-ODNs in neutrophils among foals younger than 2 months of age in that study. Because it is likely that natural infection with *R. equi* most often occurs in very early life, the objective of this study was to determine whether CpG-ODNs could activate functional responses of foals during this period. We hypothesized that *in vitro* CpG-ODN stimulation would enhance the mRNA expression of selected cytokines (interferon gamma [IFN-γ]), interleukin [IL]-4, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-23p19, IL-17, and tumor necrosis factor-alpha [TNF-α] and TLR9, as well as increase the degranulation marker β-D-glucuronidase (found in azurophilic granules) in neutrophil culture supernatants from foals between 2 and 56 days of life.

2. Materials and methods

2.1. Blood sampling and neutrophil isolation

Fourteen healthy Quarter Horse foals born at the Texas A&M University Horse Center were used in this study. Approximately 60 ml of blood were collected from foals on days 2 (within 48 h of birth), 14, and 56 of life, using 20% citrate dextrose solution as an anticoagulant. The protocol for this study was approved by the Institutional Animal Care and Use Committee of Texas A&M University. For neutrophil isolation, whole blood was allowed to settle at room temperature for 30–45 min, and then the plasma and buffy coat layers were used for neutrophil purification as described previously (Nerren et al., 2009b). The neutrophil concentration was determined using an automated cell counter (Cellometer Auto T4), and purified neutrophils were submitted to the Texas A&M University Veterinary Teaching Hospital's clinical pathology laboratory for determination of neutrophil purity (>98% of neutrophils in the cell preparations). The purified neutrophils were suspended in RPMI medium (Hyclone, UT, supplemented with 10% horse serum and 1% L-glutamine) at concentrations described below, and were subject to CpG-ODN stimulation for cytokine expression and degranulation study. On the basis of previous results (Liu et al., 2009a,b), we selected CpG 2142 (TCGCGTGCGTTTTGTCGTTTTGACGTT) to use in this study, which was generously provided to us by Merial Ltd.

2.2. Cytokine mRNA expression

Freshly isolated neutrophils (approximately 5×10^6/well) were incubated in 1 ml RPMI medium, with or without stimulation using 100 μg/ml of CpG 2142, in 12-well tissue culture plates at 37 °C in 5% CO_2 in triplicate. This CpG-ODN was selected on the basis of previous results from our laboratory (Liu et al., 2009a,b). After 4 h incubation, cells were harvested, and RNA extraction, DNase treatment, and cDNA synthesis were carried out as described previously (Liu et al., 2009b). Expression of mRNA of equine TLR9 and cytokines of different Th types (IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-23p19, IL-17, and TNF-α) was measured using real-time quantitative RT-PCR. Absolute quantification of gene copy number was conducted using the standard curve method. Equine β2-microglobulin (β2m) was used as a reference for constitutive gene expression. Standard curves were constructed for each gene, by cloning the corresponding equine cDNA gene sequence into a plasmid vector (IFN-γ, IL-12p35, IL-12p40-pCR 2.1 TOPO; IL-4, IL-10, IL-17, TNF-α, TLR9-pCR4 TOPO; IL-6, IL-8, IL-23p19-pME18S-FL3) and transforming into chemically competent *E. coli*. Plasmid DNA was extracted, and the accuracy of the cloned sequence was confirmed through bi-directional sequencing. The copy number of the plasmid standard was calculated using the following formula: copies/μl = plasmid concentration (g/μl) × 6.022×10^{23}/plasmid length (bp) × 660. A series of corresponding standards was prepared by performing 10-fold serial dilutions of cDNA clones in the range of 100 million to 0.1 copies per quantitative PCR reaction (containing 60 ng total RNA). Gene copy numbers for each target gene were determined by comparison of the fluorescence generated by each sample with standard curves of know quantities. Primer and probe sequences for each gene tested were described in Supplementary Table 1. The PCR reactions were performed in optical 384-well plates using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each 20-μl reaction mixture contained 10 μl of 2× Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen), 60 ng template cDNA, 400 nM each of forward and reverse primers, and 200 nM of TaqMan probe (Applied Biosystems). The thermal profile consisted of an initial hold at 50 °C for 2 min, followed by a single denaturation step at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, 60 °C for 60 s.

The incubation time was selected based on temporal experiments using neutrophils from adult horses (data not shown). Neutrophils were isolated using the method described above. Neutrophils (approximately 7×10^6/ml) in 1 ml of RPMI medium were incubated with medium

only, CpG 2142, or *R. equi* (ATCC 33701) for 1, 2, 4, 8, 12, and 24 h. Absolute quantification of mRNA expression for each of the aforementioned cytokines was determined for each condition (medium only, 50 μg/ml of CpG 2142, or 1×10^8 CFU/ml of *R. equi*). For each time-point, the net number of copies for stimulated samples was determined by subtracting the copy number for a given cytokine in the unstimulated sample (medium only) from that in the corresponding stimulated sample (either CpG-stimulated or *R. equi*-stimulated). A 4-h incubation period was selected because we observed no difference in copy numbers during the first 8 h for the pro-inflammatory cytokines IFN-γ and IL-6 and the chemokine IL-8, and because incubation of 12 h or longer was lowest for many cytokines (IFN-γ, IL-4, IL-6, IL-8, IL-12p35, and IL-17). Only IL-12p40 appeared to have greatest expression induced at 12 or 24 h; absolute copy numbers for this cytokine were generally very low (<10), however.

2.3. Degranulation

Neutrophil degranulation was detected by quantifying the amount of β-D-glucuronidase activity in the culture medium following stimulation of the neutrophils. Six aliquots of neutrophils suspended in RPMI medium (2×10^6 cells in a volume of 500 μl) were placed in polypropylene tubes, and divided into 2 groups (3 tubes each group). Tubes in 1 group were stimulated with CpG 2142 at 100 μg/ml final concentration, and the other group served as non-CpG stimulated control. To 1 tube for each group, opsonized live *R. equi* cells were added at a ratio of 10 bacteria to 1 neutrophil at the beginning of the incubation. Both groups were incubated for 45 min at 37 °C. After 45 min, N-formyl-methionyl-leucyl-phenylalanine (fMLP) was added (1 μM final concentration) to 1 control tube and 1 tube containing CpG 2142, and the tubes were incubated for additional 15 min, such that all tubes were incubated for 1 h. The neutrophils were then centrifuged ($250 \times g$, 10 min at 4 °C) and the supernatant (25 μl) was loaded into a black, 96-well, flat-bottom plate (Corning Costar) in quadruplicate. RPMI medium was loaded in parallel as the background control. Fifty microliters of 10 mM substrate, 4-methylumbelliferyl-β-D-glucuronide (4-MU), freshly prepared in sodium acetate buffer (pH 5.0) was added to each well and the plate was incubated at 37 °C for 4 h, protected from light. The reaction was stopped by adding 200 μl of stop solution (50 mM glycine and 5 mM EDTA). The amount of liberated 4-MU due to the β-D-glucuronidase activity was measured fluorometrically (BioTek Synergy 2, Winooski, VT) with an excitation of 355 nm and an emission of 460 nm. Using a standard curve of known concentrations of 4-MU, release of granular proteins by neutrophils was expressed as the nanomoles of 4-MU generated by 2×10^6 cells in 1 h at 37 °C.

2.4. Data analysis

For each cytokine, the ratio of mRNA expression in the stimulated sample (CpG or *R. equi*) to the unstimulated (medium) control was calculated, and exploratory data analysis was performed. When these data were not normally distributed, logarithmic ($\log_{10}$) transformation was performed to approximate a normal distribution, as assessed using the Shapiro–Wilk test statistic. Linear mixed-effects modeling was used for analysis of all mRNA expression (basal and stimulated), with individual foal modeled as a random effect to account for repeated measures over time (ages), and age was modeled as a fixed, ordered categorical effect. For CpG 2142 stimulation, when there were no significant effects of age, data were combined across all ages to examine whether there were significant effects of CpG 2142 stimulation. Linear mixed-effects modeling also was used for analysis of the degranulation data, with individual foal modeled as a random effect to account for repeated measures over time (ages), stimulus modeled as a categorical variable (with negative control as the reference group) and age modeled as a fixed, ordered categorical effect. Post hoc testing among times or stimuli was performed using the method of Sidak. A significance level of $P < 0.05$ was used for all statistical analyses, which were performed using S-PLUS (version 8.1) statistical software (TIBCO, Inc.).

3. Results

3.1. Basal expression levels of TLR9 and cytokine mRNA in foal neutrophils

Basal mRNA expression by neutrophils significantly ($P < 0.05$) increased with age for IFN-γ, IL-4, IL-6, and IL-17 (Table 1). For TNF-α, basal expression was increased on days 14 and 56 relative to day 2, but the difference relative to day 2 was only significant on day 14 (Table 1). For TLR-9, expression increased with age, but the difference was only significant between day 2 and day 56 (Table 1). Basal mRNA expression did not significantly vary with age for IL-8, IL-10, IL12-p35, IL12-p40, and IL-23p19 during the first 2 months of age (Table 1). Cytokines expressed in the greatest abundance of mRNA transcripts were IL-8 (mean expressions from 2.7 to 5.9×10^4 copies), followed by TNF-α (means from 7.4 to 10.2×10^3 copies), IL-6 (means from 2.5 to 22.2×10^3 copies), and IL-23p19 (means $(1.1–4.5) \times 10^3$ copies). Expression was very low (means of 10 or fewer copies) for IL-12 subunits p35 and p40 and IL-17.

3.2. Effect of CpG 2142 on TLR9 and cytokine mRNA expression in foal neutrophils

There was no significant difference among ages in the fold-change of mRNA expression induced by CpG 2142 except for IL-12p40 (Table 2): CpG 2142 induced significantly ($P < 0.05$) greater mRNA expression of IL-12p40 on day 56 than on days 1 or 14. Because of the absence of differences among ages, effects of CpG 2142 on mRNA expression was considered for all ages combined (Table 2). Considering all ages combined, only expression of IL-6 and IL-17 was significantly ($P < 0.05$) increased over basal expression by stimulation with CpG 2142; the mean fold-increase stimulated by CpG 2142 was 1.6 (95% confidence interval, 1.1–2.4-fold change) for IL-6 and 2.0 (1.2–3.3-fold change) for IL-17.

Table 1
Absolute basal (unstimulated) mRNA transcripts of TLR9 and cytokines (95% confidence intervals) expressed in foal neutrophils at different ages.

Gene	Mean transcript		
	Day 2	Day 14	Day 56
IFN-γ	171[a] (105–277)	627[b] (247–1587)	620[b] (291–1321)
IL-4	156[a] (61–397)	785[b] (277–2220)	841[b] (309–2294)
IL-6	1801[a] (969–3347)	7497[b] (2529–22,225)	5392[b] (2187–13,298)
IL-8	34,135[a] (23,423–49,475)	40,084[a] (27,085–59,321)	26,847[a] (16,931–42,573)
IL-10	144[a] (88–237)	261[a] (141–484)	127[a] (82–198)
IL-12p35	6[a] (4–9)	9[a] (4–19)	10[a] (7–15)
IL-12p40	3[a] (1–8)	2[a] (1–7)	2[a] (1–4)
IL-17	0.4[a] (0.3–0.6)	1.7[b] (0.8–3.9)	1.3[b] (0.8–2.3)
IL-23p19	1420[a] (970–2079)	1380[a] (599–3180)	1816[a] (1114–4531)
TNF-α	5098[a] (3082–8431)	9661[b] (7444–12,538)	8745[a,b] (7484–10,219)
TLR9	146[a] (94–225)	248[a,b] (147–418)	245[b] (172–350)

[a,b]Values in rows with different letters are significantly different ($P < 0.05$).

3.3. Effect of CpG on degranulation in foal neutrophils

Neutrophil degranulation was tested using β-D-glucuronidase as the granule marker. Release of granular proteins by neutrophils was expressed as the nanomoles of 4-methylumbelliferone generated by 2×10^6 cells in 1 h at 37 °C. There were no differences among ages in degranulation of foal neutrophils in response to the stimuli tested (Fig. 1). At all ages tested, foal neutrophils showed no response to fMLP (1 μM) in granule release (Fig. 1). Incubation with *R. equi* stimulated significant granule release in foal neutrophils ($P < 0.05$). Stimulation of foal neutrophils with CpG 2142, either alone or in combination with either fMLP (1 μM) or *R. equi*, caused significant granule release in foal neutrophils (Fig. 1). There were no significant differences in stimulation effect among the positive treatments (*R. equi*, CpG 2142, CpG 2142 with fMLP, or CpG 2142 with *R. equi*).

4. Discussion

In vitro, CpG 2142 effectively stimulated proliferation in isolated equine PBMCs (Rankin et al., 2001), and cytokine gene expression by equine PBMCs (Liu et al., 2011). Previously, our laboratory demonstrated that TLR9 mRNA is constitutively expressed in neutrophils of newborn foals and adult horses (Liu et al., 2009a). Moreover, we observed that neutrophils of 2-month-old foals and adult horses had similar relative mRNA expression of cytokines in response to CpG-ODN stimulation (Liu et al., 2009a), suggesting that the TLR9 signaling pathway in foal neutrophils is mature and could potentially be activated by CpG-ODNs at birth. This study examined the effect of age and *in vitro* stimulation with a CpG-ODN on 2 functional responses (viz., cytokine mRNA expression and degranulation [i.e., exocytosis of granular contents and the release of lytic enzymes including elastase]) of foal neutrophils at 3 time-points during the first 2 months of age.

Basal expression of several cytokines increased significantly with age. Generally, these differences were attributable to values being lower on day 2, with values on days 14 and 56 being similar to each other (Table 1). These findings suggested that there may be deficits of basal functioning of neutrophils of newborn foals relative to foals 2 weeks of age and older. For IFN-γ, IL-4, and IL-17, results of this study were consistent with findings for basal mRNA expression of these cytokines by peripheral blood mononuclear cells (PBMCs) during early life in foals (Liu et al., 2011). The extent to which decreased basal expression of these cytokines by neutrophils of newborn foals relative to foals 14 and 56 days of age reflects or contributes to maturation of different Th-type immune responses remains to be

Table 2
Mean estimated fold-change (95% confidence intervals) of mRNA gene copy numbers of TLR9 and cytokines expressed in foal neutrophils at different ages induced by CpG 2142.

Gene	Mean fold change induced by CpG			
	Day 2	Day 14	Day 56	All ages
IFN-γ	2.1[a] (0.9–4.5)	0.3[a] (0.1–1.4)	1.2[a] (0.3–5.2)	0.8 (0.6–1.5)
IL-4	3.7[a] (1.2–3.7)	0.3[a] (0.1–2.6)	4.5[a] (3.3–6.3)	1.3 (0.7–2.6)
IL-6	2.2[a] (0.8–6.1)	0.5[a] (0.1–3.0)	2.7[a] (0.6–11.5)	1.6[#] (1.1–2.4)
IL-8	0.7[a] (0.5–1.0)	0.6[a] (0.4–0.9)	0.9[a] (0.6–1.4)	0.7 (0.5–1.0)
IL-10	0.9[a] (0.6–1.2)	0.5[a] (0.2–1.2)	2.1[a] (0.9–5.1)	1.1 (0.7–1.6)
IL-12p35	1.0[a] (0.5–1.8)	1.0[a] (0.3–3.0)	2.9[a] (1.0–8.2)	1.4 (0.9–2.1)
IL-12p40	0.3[a] (0.1–0.8)	0.8[a] (0.1–4.7)	10.5[b] (1.9–57.0)	1.2 (0.6–2.7)
IL-17	1.2[a] (0.8–1.8)	ND	2.7[a] (1.1–6.8)	2.0[#] (1.2–3.3)
IL-23p19	0.7[a] (0.3–1.3)	1.7[a] (0.5–5.1)	1.6[a] (0.5–3.3)	1.2 (0.8–1.9)
TNF-α	1.0[a] (0.6–1.8)	0.5[a] (0.2–1.4)	2.5[a] (0.9–6.6)	1.1 (0.8–1.7)
TLR9	1.5[a] (0.8–2.8)	0.6[a] (0.2–1.7)	1.3[a] (0.4–3.9)	1.0 (0.7–1.4)

[a,b]Values in rows with different letters are significantly different ($P < 0.05$). ND, mean not estimated because data were available only for 1 foal at this time.
[#] CpGs induced significant ($P < 0.05$) increase in mRNA expression of cytokine.

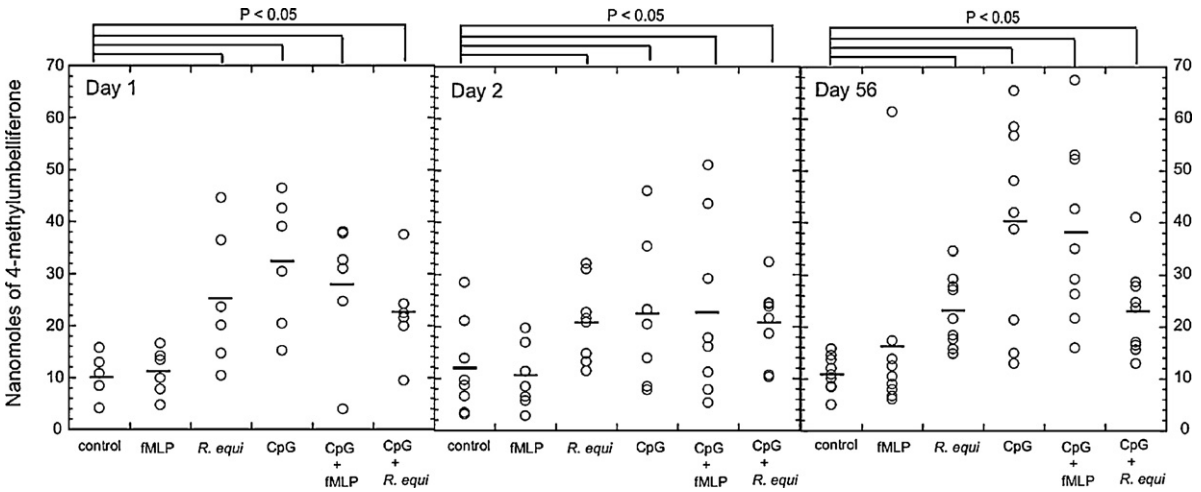

Fig. 1. Stimulation of degranulation in foal neutrophils. Neutrophils (2×10^6 cells) were incubated at 37 °C with or without stimulations. The stimulations used include fMLP (1 μM) for 15 min, *R. equi* (MOI of 10) for 1 h, CpG (100 μg/ml) for 1 h, CpG pretreatment (100 μg/ml) for 45 min followed by fMLP (1 μM) for 15 min, CpG (100 μg/ml) together with *R. equi* (MOI of 10) for 1 h. The amount of liberated 4-methylumbelliferone (4-MU) due to the beta-glucuronidase activity was measured (355/460 nm, excitation/emission) using 4-methylumbelliferyl-β-D-glucuronide as substrate. Neutrophil degranulation was expressed as the nanomoles of 4-MU generated by 2×10^6 cells in 1 h at 37 °C. Each circle represents the result from an individual foal. The mean value of the foal population was indicated as a horizontal line. Stimulation treatments that showed significant differences ($P < 0.05$ vs. control) are marked on top of the figures.

determined. Basal IL-17 mRNA expression by neutrophils increased with age, but the low copy numbers produced make it difficult to interpret the biological significance of this finding. However, the copy numbers for IL-6 were considerably larger than those for IL-17, and IL-6 is known to play a role in inducing and maintaining a Th17-type immune response.

Across all ages, CpG 2142 induced a significant ($P < 0.05$) fold-increase in mRNA expression of IL-6 by foal neutrophils. In a previous report from our laboratory, IL-6 mRNA expression was not significantly induced by CpG 2142; however, that study involved fewer foals ($N = 5$), and the tendency was for IL-6 to be increased following stimulation albeit not significantly (Liu et al., 2009a). The absence of any apparent differences among ages suggests that CpG-ODNs can induce IL-6 expression by foal neutrophils early in life. Studies to evaluate whether such stimulation can enhance innate immune resistance to infection are warranted. In addition to their role in innate immunity, evidence exists that neutrophils activated by pro-inflammatory cytokines such as IL-6 may enhance antigen presentation (Ishikawa and Miyazaki, 2005). Thus, it is possible that CpG 2142-induced expression of IL-6 may enhance both innate and adaptive immune responses in neonatal foals.

Expression of IL-17 mRNA also was induced in foal neutrophils by CpG 2142. We previously observed induction of IL-17 mRNA expression by CpG-ODNs, including CpG 2142 (Liu et al., 2011). It is difficult to know the biological importance of this statistically significant finding because the absolute copy numbers of IL-17 were very low. Further studies of the capacity of CpG 2142 to induce Th17-type cytokines and promote development of a Th17-type response by foal neutrophils are merited.

The only cytokine for which an age-related change in mRNA expression was induced by CpG 2142 was IL-12p40. The biological significance of this result is unclear because of the low absolute copy numbers of IL-12p40, the absence of effects for other cytokines examined, and the fact that this cytokine was not differentially induced by CpG 2142 (or other CpG-ODNs) in a previous report from our laboratory (Liu et al., 2009a). Thus, this observation may have occurred due to chance alone and should be interpreted with considerable caution.

Basal mRNA expression of TLR9 was generally lower for newborn foals than for foals 14 or 56 days of age; although the difference relative to day 2 of life was significant ($P < 0.05$) only at 56 days of age, the average magnitude of effect was similar at 14 days of age (mean, 248 copies; 95% CI, 147–418 copies) as day 56 (mean, 245 copies; 95% CI, 172–350 copies). Our results for changes in basal mRNA expression contrast with a previous report from our laboratory (Liu et al., 2009a); however, there were important differences between the studies. Our earlier study compared TLR9 mRNA expression between 3 foals at 1 day of age and 5 other foals at day 56 of age, whereas in this study we followed 14 foals sequentially on days 2, 14, and 56 of life. Thus, our earlier study may have lacked statistical power because of a small sample size and variation in expression levels among foals. The biological importance of this age-related variation in basal expression of TLR9 does not to appear to be high because CpG 2142 did not induce significantly greater mRNA expression at older ages for any cytokine other than IL-12p40. The difference in basal expression of TLR9 and several cytokines may simply reflect a non-specific maturational development of neutrophils of newborn foals.

Stimulation of neutrophils with CpG 2142 did not induce a significant fold-increase in mRNA expression of TLR9 at any age or across all ages combined. This finding was consistent with previous results from our laboratory for this and other CpG-ODNs evaluated using neutrophils

from 2-month-old foals (Liu et al., 2009a). This finding is contrary to results using human neutrophils for which modest stimulation of TRL9 expression was induced by CpG-ODNs (Jozsef et al., 2004). Thus, there may be species-specific variations in TLR9 expression by neutrophils in response to CpG-ODN stimulation. Although TRL9 expression was unchanged, the observation that some cytokine mRNA and degranulation responses were induced by the TLR9 agonist CpG 2142 indicates that the TLR9 signaling pathway is intact in neonatal foals.

All stimuli, including CpG 2142, induced significant ($P < 0.05$) degranulation by foal neutrophils. The finding that there were no significant differences among ages in degranulation responses to any of the stimuli indicates that neutrophils of newborn foals are functionally mature (relative to 56-day-old foals). Degranulation is an important functional response of neutrophils which plays a pivotal role in host defenses against microorganisms. Neutrophil granules contain a wide range of antimicrobial and potentially cytotoxic substances, including antimicrobial proteins, proteases, components of the oxidative respiratory burst, membrane-bound receptors for endothelial adhesion molecules, extracellular matrix proteins, and soluble mediators of inflammation (Faurschou and Borregaard, 2003). Following degranulation, these substances are released to the phagosome or to the exterior of the cell to exert antimicrobial effects. The activity of the released enzymes, such as myeloperoxidase, elastase, and β-D-glucuronidase (the azurophilic granule enzyme marker used in this study), are often used to monitor neutrophil degranulation.

We used the *Escherichia coli*-derived cell wall peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) as a stimulus for degranulation, either alone or in combination with CpG 2142. As a chemotactic agent, fMLP has been widely used in studies of neutrophil activation, due to its capability of activating all major bactericidal functions, including degranulation and superoxide generation in human neutrophils (Sengelov et al., 1993, 1994). Interestingly, we did not induce significant degranulation using fMLP with equine neutrophils, nor did we find that it modified the effects of CpG 2142 when given in combination (Fig. 1). The reason for this lack of responsiveness to fMLP is unknown. The effects of fMLP on neutrophil functions have been well studied in humans (Hess et al., 1999; Ogle et al., 1990), but evaluation of responses of neutrophils of domestic animal species are limited (Brazil et al., 1998; Franck et al., 2009; Linnekin et al., 1990; Liu et al., 2011; Sugawara et al., 1995). It was reported that fMLP was not chemotactic for either bovine (Carroll et al., 1982) or ferret (Nakata et al., 2009) neutrophils, and had no significant effect on phagocytosis by bovine neutrophils (Ducusin et al., 2001). In neutrophils from adult horses, pretreatment with LPS, TNF-α, or platelet aggregating factor (PAF) induced superoxide anion generation by lucigenin-dependent chemiluminescence (LDCL) after incubation with fMLP (Brazil et al., 1998). The same authors showed that the peak of LDCL occurred between 1 and 2 min, with complete desensitization of the response by 4 min. CpG 2142 alone failed to induce ROS by foal neutrophils, but did prime foal neutrophils to produce ROS in response to fMLP (Liu et al., 2009a). No such priming by CpG of fMLP-induced degranulation, however, was observed in this study. The effect of fMLP on chemotaxis of equine neutrophils is dose-dependent, and only a high non-physiological dose is capable of inducing a chemotactic response (Sedgwick et al., 1987; Zinkl and Brown, 1982). Conceivably, the dose of fMLP, the duration of incubation with fMLP, or both may have been inadequate to induce degranulation. It is likely that equine neutrophils may respond better to other bacterially-derived peptides. The absence of degranulation observed in this study and the stimulation of only modest ROS production in purified foal neutrophils in our previous study when using a relatively high concentration of fMLP (Liu et al., 2009a) suggests that the equine neutrophil receptor affinity for fMLP is low. Consistent with this hypothesis, the formyl peptides from *Listeria monocytogenes* and *Staphylococcus aureus* are approximately 100-fold more potent than fMLP in activating mouse neutrophils (Southgate et al., 2008).

This project had a number of limitations. First, we examined only *in vitro* effects of CpG 2142. The ability to modify functional responses of neutrophils *in vivo* is of primary clinical importance. A single incubation period of 4 h was selected for all cytokines. Although this time-period was best for most cytokines evaluated, IL-4 had peak expression after shorter incubation periods (1 or 2 h) and IL-12p40 had peak expression induced after longer incubation times (12 and 24 h). Thus, our results for IL-4 and IL-12p40 should be interpreted with this limitation in mind.

Our incubation period for degranulation may have been too long. Degranulation responses occur relatively quickly in response to stimulation (O'Flaherty et al., 1981; Sengelov et al., 1993). Thus, we may have detected even stronger degranulation responses had we examined effects following a shorter period of stimulation. Nevertheless, we documented significant and similar increases in degranulation in response to CpG 2142 by foal neutrophils at all 3 ages.

Based on previous evidence indicating effectiveness at stimulating equine neutrophils (Liu et al., 2009a,b), we selected a single B-class CpG-ODN to evaluate. It is possible that other B-class CpG-ODNs (such as CpG 2135) or C-class CpG-ODNs (such as CpG 2395), either alone or in combination, might have had stimulated stronger responses or a different cytokine profile. We lacked a positive control and a non-stimulatory CpG-ODN for our evaluation of cytokine mRNA expression. These controls would have been valuable, particularly in light of the modest stimulatory effects on mRNA expression of CpG 2142. Although we considered including these positive controls, practical limitations (number of available cells and available personnel) forced us to exclude these controls from these experiments.

The cell isolation procedures, including the reagents used, temperature fluctuations, and mechanical forces, may have activated or primed neutrophils functions (Fearon and Collins, 1983; Forsyth and Levinsky, 1990). Thus, our results must be interpreted with the understanding that unstimulated neutrophils in fact may have been partially activated. This activation may have precluded us from detecting responses to CpG-ODN stimulation by pre-activated neutrophils.

In summary, CpG 2142 stimulated Th17-type cytokine mRNA expression and degranulation by neutrophils of foals in a manner that was similar among newborn and older foals. Further evaluation of this and other CpG-ODNs for stimulating neonatal innate immune responses and promoting development of adaptive immune responses against microbial infections of neonates such as *Rhodococcus equi* is warranted.

Conflict of interest statement

The authors declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2011.11.012.

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